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Applicant: Susmu KAJIWARA et al.
Title: A DNA CHAIN USEFUL FOR INCREASING
PRODUCTION OF CAROTENOIDS
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ENGLISH TRANSLATION OF PRIORITY DOCUMENT

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

Herewith is an English language translation of the Japanese Patent Application
No. 51234/1995 filed on March 8, 1999.

Enclosed is:

[X] English Language Translation of Japanese Patent Application (47
pages).

The Commissioner is hereby authorized to charge any additional fees which may
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Respectfully submitted,

Date June 13, 2003

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DECLARATION

I, Natsuo TANAKA, of HIRAKI & ASSOCIATES, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and Japanese languages and am competent to translate from Japanese into English.
2. That I have executed, with the best of my ability, a true and correct translation into English of Japanese Patent Application No. 51234/1995 filed on March 10, 1995, a copy of which I attach herewith.

This 22nd day of May, 2003

N. Tanaka

Natsuo TANAKA



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This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application: March 10, 1995

Application Number: Japanese Patent Application
No. 051234/1995

Applicant(s): Kirin Beer Kabushiki Kaisha

March 22, 1996

Commissioner,
Patent Office

Yuji Kiyokawa (seal)

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[Title of the Invention] A DNA chain useful for increasing production of carotenoids

[Number of claims] 4

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[Name of Document] Specification 1

[Name of Document] Drawing 1

[Name of Document] Abstract 1

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[Necessity of Proof] Necessary

[Name of Invention] Specification

[Name of the Invention] A DNA chain useful for increasing
production of carotenoids

[Scope of the Claim]

[Claim 1] A DNA chain having characteristic of increasing
carotenoid production, and containing the nucleotide sequence
which encodes the polypeptide having the amino acid sequence
substantially described in SEQUENCE ID No. 1, or a DNA chain
which hybridizes with said DNA chain.

[Claim 2] A DNA chain having characteristic of increasing
carotenoid production, and containing the nucleotide sequence
which encodes the polypeptide having the amino acid sequence
substantially described in SEQUENCE ID No. 2, or a DNA chain
which hybridizes with said DNA chain.

[Claim 3] A method for producing carotenoid characterized by
introducing DNA chain described in one of claim 1 or 2 into
carotenoid-producing microorganisms, culturing said transformed
microorganism and obtaining higher carotenoid content in the
culture broth and cells .

[Claim 4] A method for producing carotenoid characterized by
introducing DNA chain containing the nucleotide sequence which
encodes the polypeptide having the amino acid sequence
substantially described in SEQUENCE ID No. 3, or DNA chain which
hybridizes with said DNA chain introducing to carotenoid-
producing microorganism, culturing said transformed microorganism
and obtainig higher carotenoid content in the culture broth and
cells .

[Detailed Description of the Invention]

[Field of the Invention]

The present invention relates to a DNA chain which provides higher carotenoid content during biosynthesis of carotenoid and a method for producing carotenoids characterized by introducing said DNA chain into carotenoid producing microorganism to express said chain and to obtain higher carotenoid content.

[Prior Art]

Carotenoid is a general name of a kind of natural pigments. Generally, carotenoids have 40 carbon atoms and consists of isoprene skeletons, and Carotenoids are abundant in the natural world. Approximately 600 kinds of carotenoids have been isolated and identified up to the present [(see Key to carotenoids. Basel-Boston, Birkhauser, 1987(Pfander, H. ed.))]. Carotenoids are synthesized through the isoprenoid biosynthetic pathway, a part of which is common to the pathways for steroids and other terpenoids. Passing through the isoprene common biosynthetic pathway, hydroxymethylglutaryl-CoA(HMG-CoA) is converted to isopentenyl pyrophosphate(IPP), which has 5 carbon atoms, via mevalonate. Then IPP is converted to dimethylallyl pyrophosphate(DMAPP) by isomerization. Then, by polycondensation with IPP which has 5 carbon atoms, DMAPP is converted sequentially to geranyl pyrophosphate(GPP which has 10 carbon atoms), farnesyl pyrophosphate(FPP which has 15 carbon atoms), geranylgeranyl pyrophosphate(GGPP which has 20 carbon atoms) and so forth (Figure 1).

The carotenoid biosynthetic pathway is branched from the isoprene common pathway at the point of GGPP is formed. At the point, two molecules of GGPP are condensed to synthesize phytoene which is the first carotenoid and colorless. Then, phytoene is converted to lycopene by desaturation reaction. Then, lycopene

is converted to β -carotene by cyclization. Various xanthophylls such as zeaxanthin and astaxanthin are synthesized by introducing hydroxyl groups or keto groups to β -carotene.

Recently, the inventors of the present invention cloned the carotenoid biosynthesis genes derived from Erwinia uredovora, which is a non-photosynthetic epiphytic bacterium in Escherichia coli by using yellowish color of Er. uredovora as markers and elucidated the functions of the genes. Then, various combinations of these genes are introduced to express, and it made possible that microorganisms such as E. coli and yeast produce phytoene, lycopene, β -carotene, zeaxanthin and so forth(See Figure 2): [See Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K., "Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli", J. Bacteriol., 172: 6704-6712 (1990); Misawa, N., Yamano, S., and Ikenaga, H., "Production of β -carotene in Zymomonas mobilis and Agrobacterium tumefaciens by introduction of the biosynthesis genes from Erwinia uredovora", Appl. Environ. Microbiol., 57: 1847-1849 (1991); Yamano, S., Ishii, T., Nakagawa, M., Ikenaga, H., and Misawa, N., "Metabolic engineering for production of β -carotene and lycopene in Saccharomyces cerevisiae", Biosci. Biotech. Biochem., 58: 1112-1114 (1994) and Japanese Patent Application laid-open No. HEI 3-58786(Japanese Patent Application filing No. HEI 2-53255):"A DNA chain useful for synthesis of carotenoids" by the inventors of the present invention]. With the carotenoid biosynthesis genes from Er. uredovora, carotenoids can be synthesized from FPP. Since FPP is the common substrate not only for carotenoids but also for

steroids and other terpenoids, bacteria incapable of synthesizing carotenoids also have FPP. Accordingly, for example, when four crt genes, crtE, crtB, crtI and crtY, which are necessary for biosynthesis of β -carotene from FPP are introduced in microorganisms, the microorganism becomes capable of producing β -carotene (See Figure 2). Furthermore, by the same procedures as mentioned above, the inventors cloned the carotenoid biosynthesis genes derived from a marine bacterium, Agrobacterium aurantiacum in E. coli. By expressing various combinations of the genes from the bacterium and those from the above-mentioned Er. uredovora, it made possible that the microorganisms such as E. coli produce astaxanthin, canthaxanthin and so forth (See Figure 3): (Norihiro Misawa et al., "Elucidation of an astaxanthin biosynthetic pathway at the level of the biosynthesis genes", Abstract of the 36th Symposium on the chemistry of natural products: 175-180 (1994)). Among the above carotenoids, astaxanthin, zeaxanthin and β -carotene are already in practical use and are regarded as promising substances. They are used for food or feed additives as red or yellow natural coloring agents or as nutritional aid having cancer prophylactic activity, immunopotentiating activity or provitamin A activity. Accordingly, when the carotenoid biosynthesis genes obtained by the inventors is used as exogenous genes for transforming microorganisms such as E. coli to express, it gave microorganisms such as E. coli the capability of biosynthesis for producing useful carotenoids. Up to now, it is the only way to improve production of useful carotenoids was to find out microorganism which can synthesize sufficient amount of a targeted carotenoid, and to try to increase its production by investigating culture conditions or mutation treatment. Owing to

the studies done by the inventors, it became possible to choose host microorganism which is cultured easily and proliferates rapidly, and is guaranteed to be safe for food regardless of its carotenoid producing capability. As a matter of course, it is also possible to use microorganisms which can synthesize sufficient amount of useful carotenoids originally. In such a case, by transforming the microorganisms with carotenoid biosynthesis genes, it became possible to obtain higher carotenoid production or to alter final carotenoid products. For example, when both crtW and crtZ genes from Ag. aurantiacum were introduced into a microorganism capable of producing β -carotene as a final product to express them, the microorganism was transformed to another one which produce astaxanthin as a final product.

On the other hand, both astaxanthin and β -carotene can also be synthesized by organic synthesis methods. In these cases, considering these carotenoids are used for feed or food additives, there is problems that by-products are also produced and such synthetic products are not preferred by consumers because they prefer natural products. However, carotenoids produced by the conventional fermentation methods could not compete with those by the organic synthesis methods in price. As mentioned earlier, when the above mentioned carotenoid biosynthesis genes are used, it improves the fermentation methods, thereby it is considered that the carotenoid produced by the fermentation methods will be able to compete with those by the organic synthesis methods in price. If the microorganism can accumulate enough amount of carotenoid in itself, the carotenoid produced by the microorganisms will succeed in such price competition. Therefore,

a technology to obtain higher carotenoid content by using microorganisms has been longed for.

Until now, in order to obtain higher carotenoid production in its biosynthesis; the traditional random mutation method is only employed to select mutant strains having higher carotenoid content with mutagenic agent such as NTG. However, this method requires huge amount of time and labor of technicians. In addition, even if enhancement of carotenoid synthesis is successfully achieved, the method requires both huge amount of time and effort to inhibit decreasing of carotenoid content caused by frequent reverse mutations naturally happens because the method lacks its theoretical basis.

[The Problems to Be Solved by the Invention]

The object of the present invention is to increase amount of carotenoids biosynthetically produced by microorganisms.

[Means to Solve the Problems]

To solve the above problem, the inventors have investigated the problem thoroughly and developed a novel technology which provides several times higher carotenoid production amount by introducing a DNA chain containing only one gene into a carotenoid producing microorganism to express the gene in them.

More specifically, the inventors of the present invention found the followings and completed the present invention. When a DNA chain containing a gene substantially encoding an amino acid sequence of IPP isomerase which converts IPP into DMAPP, is introduced in microorganisms such as E. coli having carotenoid synthesis gene derived from Er. uredovora and so forth, content of carotenoid in cells such as lycopene and β -carotene becomes 1.5-4.5 times higher than that in control cells can be achieved.

The gene substantially encoding IPP isomerase amino acid sequence which converts IPP into DMAPP was obtained from the astaxanthin producing microorganisms such as Phaffia rhodozyma and Haematococcus pluvialis.

The characteristics of the DNA chain of the present invention are as follows.

(1) A DNA chain capable of increasing carotenoid production amount and containing the nucleotide sequence which encodes the polypeptide having the amino acid sequence substantially shown in Sequence ID No. 1, or a DNA chain that can be hybridized with said DNA chain.

(2) A DNA chain capable of increasing carotenoid production and containing the nucleotide sequence which encodes the polypeptide having the amino acid sequence substantially shown in Sequence ID No. 2, or a DNA chain that can be hybridized with said DNA chain.

The present invention also relates to a method for carotenoid production. The characteristics of the carotenoids production methods of the present invention are as follows.

(3) A production method characterized by introducing the DNA chain mentioned above either (1) or (2) into carotenoid producing microorganism, culturing said transformed microorganism and increasing carotenoid content in the cells and culture broth.

(4) A production method characterized by introducing the DNA chain containing the nucleotide sequence which encodes the polypeptide having the substantially same amino acid sequence shown in Sequence ID No. 3, or a DNA chain that can be hybridized with said DNA chain into carotenoid producing microorganism, culturing said microorganism and increasing carotenoid content in the cells and culture broth.

The present invention is described herein below.

As described in before, by introducing the carotenoid biosynthesis gene derived from microorganisms such as Erwinia uredovora, the non-photosynthetic soil bacteria and Agrobacterium aurantiacum, the marine bacteria) into other microorganisms which do not produce carotenoids such as E. coli, the microorganism can produce useful carotenoids such as astaxanthin, zeaxanthin, β -carotene and lycopene. In order to compete in price of the carotenoid produced by using the organic synthesis methods, it is necessary to achieve as higher carotenoid production as possible. The IPP isomerase gene, which include the gene encoding the polypeptide whose amino acid sequence is substantially IPP isomerase, of the invention is extremely useful for increasing the production amount of carotenoids. By using modern biotechnology, it is relatively easy to increase production amount of a protein encoded by an exogenous gene by enhancing expression level of the gene. However, if amounts of substrate necessary for a protein, that is enzyme, is limited, higher production of the protein does not lead to higher production of biochemicals such as carotenoids. For example, without sufficient amount of FPP, which is the first substrate, enhancement of expression level of the carotenoid synthesis genes does not lead to higher amount of carotenoids production. This time, we succeeded in increasing carotenoid production amount by introducing the IPP isomerase gene. It is considered that the introduction of the IPP isomerase gene makes the flow of, the upstream of the pathway up to FPP larger (more efficient) and consequently, increased supply of FPP led to higher carotenoid production amount. The present invention started from the

findings that by introducing either the gene encoding IPP isomerase, which convert from IPP to DMAPP vise versa, or encoding the protein homologous to IPP isomerase into carotenoid producing microorganism such as E. coli, to express the gene, carotenoid production amount is increased. By using carotenoid biosynthesis genes from Er. uredovora, cDNA expression libraries of Phaffia rhodozyma, Haematococcus pluvialis and so forth were prepared in β -carotene producing E. coli as a host. As increased β -carotene content in E. coli made, some of the yellowish colonies brighter till almost orange. The plasmids extracted from such E. coli colonies were analyzed and were found to have genes with high homology to IPP isomerase of Saccharomyces cerevisiae. It has been speculated that HMG-CoA reductase(Figure 1), which catalyzes the reaction from HMG-CoA to mevalonate, may be the rate limiting enzyme for terpenoids including carotenoids. However, as for IPP isomerase, any such report has not been presented. Therefore, increase of carotenoid production by introducing a IPP isomerase gene was a new finding.

The present invention provides a DNA chain having characteristics of increasing carotenoid production amount, and it containing the nucleotide sequence which encodes the polypeptide having the substantially same amino acid sequence as those of IPP isomerase, and a production method for carotenoid characterized by introducing said DNA chain into the carotenoid producing microorganism, culturing said transformed microorganism and increasing carotenoid content in the culture broth and cells.

The DNA chains of the present invention includes the DNA chains mentioned above (1) or (2), or the DNA chains which hybridize to said chains under stringent conditions.

Substantially, the polypeptides encoded by the DNA chains of the present invention have the amino acid sequences shown in SEQUENCE ID No. 1(A-B in Figure 4) or in SEQUENCE ID No. 2(C-D, in Figure 5). In the present invention, the polypeptides encoded by these DNA chains, the proteins of which amino acid sequence is substantially IPP isomerase, may be altered by deletion, replacement, addition and so forth of some amino acids, as long as the resulted polypeptides hold their higher carotenoid production activity. This allowance corresponds to "having the substantially same amino acid sequence substantially shown in SEQUENCE ID No. 1 or No. 2". As an example, a sequence which lacks the first amino acid(Met) can be included as the altered polypeptide or the altered enzyme. Needless to say, the DNA chains of the present invention include not only the chains having the nucleotide sequences which encode the amino acid sequences shown in SEQUENCE ID No. 1 and 2(Figures 4 and 5), but also the degenerate isomers of the chains, which differs only on degenerate codons and encode the same polypeptides as the original chains do.

(1) Obtaining the DNA chains

One method to obtain a DNA chain having the nucleotide sequence which encodes the amino acid sequence of the above protein is chemical synthesis of the DNA chain at least a part of the chain according to the known nucleic acid synthesis method. However, considering that there are so many amino acids bound in the protein, it would be more preferable than chemical synthesis to make cDNA libraries of Haematococcus pluvialis or Phaffia rhodozyma or the like to obtain a targeted DNA chain by applying

some popular method in the field of genetic engineering such as hybridization with appropriate probes.

(2) Transformation of microorganisms such as E. coli and expression of gene

Higher carotenoid content in culture broth or cells of microorganisms can be achieved by introducing the above mentioned DNA chain of the present invention into appropriate microorganisms such as carotenoid-producing bacteria such as E. coli and Zymomonas mobilis containing carotenoid biosynthesis genes from Erwinia uredovora and so forth, or carotenoid-producing yeast such as Saccharomyces cerevisiae containing carotenoid biosynthesis genes from Erwinia uredovora and so force.

The outline of the method to introduce exogenous genes into preferable microorganisms is mentioned below.

Procedures or methods to introduce and express exogenous genes in microorganisms such as E. coli, besides those mentioned below in the present invention, includes those widely used in the field of genetic engineering. Those are applicable to the invention. See "Vectors for cloning genes", Methods in Enzymology, 216: 469-631 (1992), Academic Press; "Other bacterial systems", Methods in Enzymology, 204: 305-636 (1991) Academic Press).

[E. coli]

There are some established and efficient methods to introduce exogenous genes to E. coli such as Hanahan's method and rubidium method, and they are applicable to the present invention (See Sambrook, J., Fritsch, E. F., Maniatis, T., "Molecular cloning-A laboratory manual", Cold Spring Harbor Laboratory Press (1989)). Expression of exogenous genes in E. coli can be performed by

known methods (See "Molecular cloning-A laboratory manual",
ibid.), for example, vectors for E. coli such as pUC and
pBluescript vectors having lac promoter can be used. The
inventors of the present invention used pSPORT1 vector or
pBluescript II KS vector having lac promoter as vectors for E.
coli, and inserted the IPP isomerase gene, derived from
Haematococcus pluvialis, Phaffia rhodozyma or Saccharomyces
cerevisiae, into the lac promoter with the direction of reading
through of the transcription, and expressed the gene in E. coli.
[Yeast]

There are some established methods such as the lithium method
to introduce exogenous genes into Saccharomyces cerevisiae, yeast,
and such methods are applicable to the present invention (See
"New biotechnology on yeast", Ed. Bio-industry Association(Yuichi
Akiyama, editor in chief), Igaku Syuppan Center). Expression of
exogenous genes in yeast can be performed as follows. Using both
promoters and terminators, e.g. for PGK and GPD, an expression
cassette is constructed by inserting the exogenous gene so that
during transcription, the gene is to be read through at the
position between the promoter and the terminator. Expression can
be performed by inserting the expression cassette into a vector
for S. cerevisiae such as YRp vectors (multi-copy vectors for
yeast, replication starts at ARS sequence of yeast chromosome),
YE_p vectors (multi-copy vectors for yeast, replication starts at
2 μ m DNA) and YIp vectors (vectors for yeast chromosome, no
starting point of replication in yeast) (See "New biotechnology
on yeast", ibid.; "Genetic engineering for production of
substances", Ed. Japanese Society of Agrochemical Chemistry,
Asakura Publishing company; or Yamano, S., Ishii, T., Nakagawa,

M., Ikenaga, H., Misawa, N., "Metabolic engineering for production of β -carotene and lycopene in Saccharomyces cerevisiae", Biosci. Biotech, Biochem., 58: 1112-1114 (1994)).
[Zymomonas mobilis]

Introduction of exogenous genes into Zymomonas mobilis, the ethanol-producing bacterium can be performed by conjugal transfer method which is commonly used for gram negative bacteria. Expression of exogenous gene in Zymomonas mobilis can be performed by using pZA22 vector for this bacterium (See Katsumi Nakamura, "Molecular breeding of Zymomonas bacteria", Journal of the Japanese Society of Agrochemical Chemistry, 63: 1016-1018 (1989); and Misawa, N., Yamano, S., Ikenaga, H., "Production of β -carotene in Zymomonas mobilis and Agrobacterium tumefaciens by introduction of the biosynthesis genes from Erwinia uredovora", Appl. Environ. Microbiol., 57: 1847-1849 (1991)).

(3) Method to increase carotenoid production in microorganisms

By applying the above mentioned procedures or methods for introduction and expression of exogenous genes in microorganisms, both the carotenoid synthesis genes and the IPP isomerase gene can be introduced to express, and microorganisms capable of producing large amount of carotenoid can be obtained.

Farnesyl pyrophosphate (FPP) is the common substrate not only for carotenoids but also for other terpenoids such as sesquiterpenes, triterpenes, sterols and hopanols. In general, since microorganisms are synthesizing terpenoids even though they are not capable of synthesizing carotenoids, basically all of the microorganisms possesses FPP as an intermediate metabolite. On the other hand, Erwinia uredovora, the non-photosynthetic bacterium having the carotenoid synthesis genes can synthesize up

to several useful carotenoids such as lycopene, β -carotene, zeaxanthin by using FPP as a substrate. When the genes are combined with the carotenoid synthesis genes of Agrobacterium aurantiacum, the marine bacterium, up to several useful carotenoids such as cantaxanthin and astaxanthin can also be synthesized (See Figures 2 and 3). The inventors of the present invention already confirmed that by introducing crt genes of Erwinia uredovora into microorganisms such as Saccharomyces cerevisiae, yeast and Zymomonas mobilis, ethanol-producing bacteria; these microorganisms can produce carotenoids such as β -carotene as anticipated [Yamano, S., Ishii, T., Nakagawa, M., Ikenaga, H., Misawa, N., "Metabolic engineering for production of β -carotene and lycopene in Saccharomyces cerevisiae", Biosci. Biotech, Biochem., 58:1112-1114 (1994); Misawa, N., Yamano, S., Ikenaga, H., "Production of β -carotene in Zymomonas mobilis and Agrobacterium tumefaciens by introduction of the biosynthesis genes from Erwinia uredovora", Appl. Environ. Microbiol., 57:1847-1849 (1991); and Japanese laid-open Patent Application No. HEI 3-58786(Japanese Patent Application filing No. HEI 2-53255):"A DNA chain useful for synthesis of carotenoids" by the inventors].

From the above findings, it can be expected that when an appropriate combinations of the carotenoid synthesis genes derived from Er. uredovora and those from marine bacteria(typically the carotenoid synthesis genes derived from Ag. aurantiacum) are introduced into the same microorganism simultaneously, as a principle, all of the microorganisms, in which such genes are introduced and of which introduction-

expression system is established, can produce useful carotenoids such as astaxanthin and zeaxanthin.

In such cases, if the IPP isomerase gene (typically, derived from Haematococcus pluvialis, Phaffia rhodozyma and Saccharomyces cerevisiae) is introduced according to the above mentioned method, and is expressed concomitantly with the above carotenoid synthesis gene, higher production amount of useful carotenoids can be achieved.

(4) Deposit of the microorganisms

The recombinant E. coli strain JM109 has been deposited as follows with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology. The strain contains the plasmid having the isolated gene which is the DNA chain of the invention. The names of the plasmids are shown in the parentheses.

(i) JM109(pRH1)

Deposit No.: FERM BP-5032

Date of Receipt: March 6th, 1995

(ii) JM109(pHP11)

Deposit No.: FERM BP-5031

Date of Receipt: March 6th, 1995

(ii) JM109(pSI1)

Deposit No.: FERM BP-5033

Date of Receipt: March 6th, 1995

[Example]

The following examples illustrate the present invention in more detail, however, the present invention is not limited to them. The genetic recombination experiments used here are based on the standard methods (Sambrook, J., Fritsch, E. F., Maniatis,

T., "Molecular cloning-A laboratory manual", Cold Spring Harbor Laboratory Press (1989)) unless otherwise stated.

(EXAMPLE 1) Biological materials and culture conditions

Phaffia rhodozyma ATCC 24230 strain(Astaxanthin-producing yeast) registered at the American Type Culture Collection(ATCC) is used. YM media(yeast extract 0.3%, malt extract 0.3%, bactopectone 0.5%, Glucose 1%) is used for Ph. rhodozyma. Haematococcus pluvialis, the astaxanthin-producing green alga, NIES-144 strain registered at the Global Environmental Forum is used. Ha. pluvialis is cultured at 20°C for about 4 days in basic culture media(yeast extract 0.2%, sodium acetate 0.12%, L-asparagin 0.04%, magnesium chloride hexahydrate 0.02%, ferrous sulfate heptahydrate 0.001%, calcium chloride dihydrate 0.002%) under 12 hr light(20 $\mu\text{E}/\text{m}^2\text{s}$)/12 hr dark condition. Furthermore, in order to induce astaxanthin synthesis in Ha. pluvialis, cyst formation, a kind of differentiation, has to be induced. To induce cyst formation, both acetic acid 45 mM and ferrous sulfate heptahydrate 450 μM at final concentrations are added. Ha. pluvialis in the media is cultured for about 12 hr at 20°C with light(125 $\mu\text{E}/\text{m}^2\text{s}$). Saccharomyces cerevisiae(Yeast for laboratory use) S288C strain registered at the Yeast Genetic Stock Center is used. For Sa. cerevisiae, YPD media(yeast extract 1%, bactopectone 2%, glucose 2%) is used.

(EXAMPLE 2) Preparation of whole RNA in Phaffia rhodozyma

Phaffia rhodozyma ATCC 24230 strain is cultured with shaking for approx. 24 hr at 20°C in 400 ml of YM media. When the turbidity of the media reached at $\text{OD}_{600} = 0.4$, the bacteria are collected and frozen in liquid nitrogen. The frozen bacteria are

stored in the freezer at -80°C and used for preparing total RNA. After thawing the frozen bacteria in a tube on ice, the bacteria are suspended in 6 ml of ANE buffer (10 mM sodium acetate, 100 mM sodium chloride, 1 mM EDTA, pH 6.0). Glass beads are added to cover the surface of the bacteria layer. Then, 600 μl of 10% SDS and 6 ml of phenol prewarmed at 65°C are added. The suspension is kept at 65°C for 5 minutes, and the tube is vortexed to crush cell membranes at every 30 seconds. Then, the suspension is rapidly cooled down to room temperature and centrifuged for 10 minutes at $1,500 \times g$ at room temperature. Equal volume of phenol is added to the supernatant and vortex for 2 minutes. Then the suspension was centrifuged for 10 minutes at $1,500 \times g$ at room temperature. Then, by using equal volume of phenol/chloroform (1/1 (v/v)) and chloroform alone, the same procedures as above are performed. To the resulted supernatant, one tenth volume of 3 M sodium acetate and three volume of ethanol are added; then the supernatant is stored in the freezer at -20°C for 30 minutes. The supernatant is centrifuged for 15 minutes at $15,000 \times g$ at 4°C , a pellet is rinsed with 70% ethanol and dried. The residual is dissolved in 200 μl of sterilized water to make total RNA solution of Ph. rhodozyma. By this preparation procedure, 1.6 mg of total RNA is obtained.

(EXAMPLE 3) Preparation of whole RNA in Haematococcus pluvialis
Haematococcus pluvialis NIES-144 strain is cultured for approx. 4 days in 800 ml of the basic culture media under the condition of 20°C , light intensity at $20 \mu\text{E}/\text{m}^2\text{s}$ and 12 hr light/12 hr dark cycle. Then, both acetic acid 45 mM and ferrous sulfate heptahydrate 450 μM as final concentrations are added. The H.

pluvialis in the media is cultured for approx. 12 hr at 20°C with light(125 $\mu\text{E}/\text{m}^2\text{s}$). The bacteria are collected from the media, frozen in liquid nitrogen and crushed in the mortar to give powder. Then, three ml of ISOGEN-LS[Nippon Gene K.K.] is added to the powder and stand for 5 minutes. Then 0.8 ml of chloroform is added, and the solution is stirred vigorously for 15 seconds and stand at room temperature for 3 minutes. The solution is centrifuged for 15 minutes at 4°C, 12,000 x g, two ml of isopropanol is added to the supernatant and the supernatant is stood at room temperature for 10 minutes. Then, the solution is centrifuged for 10 minutes at 4°C, 12,000 x g. The resulted pellet is rinsed with 70% ethanol to dry. After drying, the residual is dissolved in 1 ml of TE buffer(10 mM Tris-HCl pH 8.0, 1 mM EDTA) to make total RNA solution of Ha. pluvialis. By this preparation procedure, 4.1 mg of whole RNA was obtained.

(EXAMPLE 4) Establishing cDNA expression libraries of Phaffia rhodozyma and Haematococcus pluvialis

By using Oligotex-dT30 Super[Takara Syuzo K.K.], poly A + RNA from Phaffia rhodozyma and Haematococcus pluvialis are purified from approx. 1 mg total RNA respectively. The purification is performed according to the methods mentioned in the package insert. By following the method, approx. 26 μg of poly A + mRNA from Ph. rhodozyma and approx. 14 μg of it from Ha. pluvialis are purified.

Preparation of cDNA is performed with SuperscriptTM plasmid system(GIBCO BRL) by the method mentioned in the package insert with some modifications. Approx. 5 μg of poly A + mRNA is used. A synthetic DNA consisting of the recognition sequence for the

restriction enzyme NotI and 15 mers oligo-dT is used as a primer. The complementary DNA is synthesized with reverse transcriptase, SUPERScript RT. Then, by using Escherichia coli DNA ligase, E. coli DNA polymerase and E. coli RNase H, double strand DNA is synthesized. Then, the linker of the restriction enzyme SalI is bound by using T4 DNA ligase. cDNA is designed to have the SalI site at the upstream terminal of itself and the NotI site at the downstream of poly A. Fractionation by size of these cDNAs is performed by electrophoresis and the fractions ranging from 0.7 kb to 3.5 kb are collected. cDNA in the collected fractions is ligated to cDNA expression vector pSPORT I NotI-SalI-Cut by using both the ligation buffer which is included in the kit, 50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% PEG 8,000 and T4 DNA Ligase. The cDNA expression vector pSPORT I has lac promoter at the upstream of the SalI site and can express cDNA in E. coli. Then, by using whole the ligated DNA solution, transformation of the competent cells of E. coli DH5 α prepared is performed according to the method described in "Molecular Cloning 2nd edition: Cold Spring Harbor Laboratory, 1.21-1.41(1989). Approx. 200,000 transformed strains of Ph. rhodozyma and approx. 40,000 transformed strains of Ha. pluvialis are obtained. After collecting all of the transformants, the plasmid DNA is prepared according to the method described in "Molecular Cloning 2nd edition, *ibid.*" As a result, 0.9 mg and 0.6 mg of plasmid DNAs are obtained respectively and these are assigned as cDNA libraries of Ph. rhodozyma and Ha. pluvialis.

(EXAMPLE 5) Preparation of carotenoid-producing E. coli

The plasmid pCAR16(Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K., Harashima, K., "Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli", J. Bacteriol., 172:p.6704-6712 (1990) and Japanese Patent Application laid-open No. HEI 3-58786 (Japanese Patent Application filing No. HEI 2-53255): "A DNA chain useful for synthesis of carotenoids" by the present inventors) having the carotenoid synthesis genes except for crtZ derived from Erwinia uredovora, is digested with BstEII, treated with Klenow enzyme and religated to inactivate the crtX gene by frame shift. After that, the 6.0 kb Asp718(KpnI)-EcoRI fragment containing crtE, crtB, crtI and crtY genes necessary for β -carotene production is taken out. The fragment is then inserted into the EcoRV sites of the E. coli vector pACYC184 and the desirable plasmid(named pACCAR16 Δ crtX, FIGURE 7) is obtained. E. coli containing this plasmid (pACCAR16 Δ crtX) is chloramphenicol resistant and has yellowish color due to β -carotene production.

Then, the plasmid pCAR16 is digested with BstEII/SnaBI, treated with Klenow enzyme and religated to remove the 2.26 kb BstEII-SnaBI fragment containing crtX and crtY genes. After that, the 3.75 kb Asp718(KpnI)-EcoRI fragment containing crtE, crtB and crtI genes necessary for lycopene production is taken out. The fragment is then inserted into the EcoRV sites of the E. coli vector pACYC184 and the desirable plasmid(named pACCRT-EIB, FIGURE 7) is obtained. E. coli containing pACCRT-EIB is chloramphenicol resistant and has reddish color due to lycopene production (Cunningham Jr., F. X., Chamovitz, D., Misawa, N., Gatt, E., Hirschberf, J., "Cloning and functional expression in

Escherichia coli of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of β -carotene", FEBS Lett., 328: 130-138 (1993)).

Then, the plasmid pCAR16 is digested with BstEII/Eco52I, treated with Klenow enzyme and religated to remove the 3.7 kb BstEII-Eco52I fragment containing crtX, crtY and crtI genes. After that, the 2.3 kb Asp718(KpnI)-EcoRI fragment containing crtE and crtB genes (FIGURE 2) necessary for phytoene production is taken out. The fragment is then inserted into the EcoRV sites of the E. coli vector pACYC184 and the decibel plasmid (named pACCRT-EB, FIGURE 7) is obtained. E. coli containing pACCRT-EB is chloramphenicol resistant and does not show color change as phytoene is colorless (Linden, H., Misawa, N., Chamovitz, D., Pecker, I., Hirschberg, J., Sandmann, G., "Functional complementation in Escherichia coli of different phytoene desaturase genes and analysis of accumulated carotenes", Z. Naturforsch. 46c: 1045-1051 (1991)).

(EXAMPLE 6) Screening of genes that increase β -carotene production

As the E. coli strain JM101 containing the above plasmid pACCAR16 Δ crtX shows yellowish color due to β -carotene production, it was investigated whether more yellowish transformant can be obtained by introducing cDNA expression library of Phaffia rhodozyma or Haematococcus pluvialis. As a first step, competent cells of E. coli JM101 containing pACCAR16 Δ crtX are prepared according to the method described in "Molecular cloning 2nd edition: Cold Spring Harbor Laboratory, 1.21-1.41(1989). Then, one hundred ng of each cDNA expression library of Ph. rhodozyma

and Ha. pluvialis is introduced to 1 ml of the competent cells. Approx. 200,000 transformants of Ph. rhodozyma and approx. 40,000 transformants of Ha. pluvialis are obtained and inoculated for screening on the LB plate (bactotrypton 1%, yeast extract 0.5%, NaCl 1%, agar 1.5%) containing 150 µg/ml of ampicillin, 30 µg/ml of chloramphenicol and 1 mM of IPTG. From the screening, 5 strains of Ph. rhodozyma and 10 strains of Ha. pluvialis shows deep yellowish color than other strains and they are isolated. The plasmid DNA extracted from these strains is subject to restriction enzyme analysis, and it was found that the plasmids from the five strains and ten strains have common DNA fragment respectively. Of these screened plasmids derived from the cDNA expression libraries, a plasmid from Ph. rhodozyma was named pRH1 (Figure 8) and another plasmid from Ha. pluvialis was named pHP1. In addition to that, a fragment is taken out after digesting pHP1 with SalI and NotI, and then, the fragment is inserted into pBluescript KS+. The resulted plasmid was named pHP11 (FIGURE 8) and was used for the experiments mentioned below.

(EXAMPLE 7) Nucleotide sequence determination on the gene that increases β-carotene production

From the plasmids pRH1 and pHP1, the deletion plasmids which lack various lengths from the original plasmids are prepared by the following procedures. By using those deletion plasmids, the nucleotide sequences are determined. Decomposition of pRH1 is performed with both EcoRI and PstI, or with both NotI and SphI. Decomposition of pHP1 is performed with both AatII and BamHI, or with both KpnI and EcoRI. After extraction with phenol/chloroform, DNA is recovered by ethanol precipitation.

Each DNA fraction is then dissolved in 100 μ l portions of ExoIII buffer(50mM Tris-HCl, 100mM NaCl, 5mM MgCl₂, 10mM 2-mercaptoethanol, pH 8.0) and is kept at 37°C after addition of 180 units of ExoIII nuclease. Ten μ l portions of the solution are sampled every 30 seconds and transferred to tubes containing 10 μ l of MB buffer(40 mM NaCl, 2 mM ZnCl₂, 10% glycerol, pH 4.5) in an ice bath. After sampling, the 10 tubes are kept at 65°C for 10 minutes to inactivate the enzyme. Then, 5 units of mung bean nuclease is added and kept at 37°C for 30 minutes. From one original plasmid, ten different kind of DNA fragments are recovered by agarose gel electrophoresis. The degree of deletion of each fragment varies. The terminals of the recovered DNAs are smoothed with Klenow enzyme to subject to ligation reaction at 16°C overnight, and by using resulting DNA, E. coli DH5 α is transformed to obtain clones. The plasmids are prepared from the various clones obtained, and nucleotide sequences are determined by using luminescence primer cycle sequence kit(Applied Biosystems corp.) with an automatic sequencer.

As a result, it was found that the nucleotide sequence of the cDNA in pRH1 derived from Phaffia rhodozyma consists of 1,099 base pairs (SEQUENCE ID No. 4), and there is an open reading frame which encodes a polypeptide having 251 amino acids (which corresponds the region from A to B in Figures 4). It was also found that the nucleotide sequence of the cDNA in pHP1 derived from Haematococcus pluvialis consists of 1,074 base pairs (SEQUENCE ID No. 5), and there is an open reading frame which encodes a polypeptide having 259 amino acids (which corresponds the region from C to D in Figure 5). The amino acid sequences expected from these open reading frames are investigated by

analyzing homology in the Gene Bank. Both of the amino acid sequences of Ph. rhodozyma and Ha. pluvialis have significant homology with the IPP isomerase gene of Saccharomyces cerevisiae, 27.0% for Ph. rhodozyma and 20.3% for Ha. pluvialis. Therefore the genes were identified as the IPP isomerase gene.

(EXAMPLE 8) Preparation of total DNA in Saccharomyces cerevisiae

Preparation of total DNA in Saccharomyces cerevisiae is performed according to the method described in "Methods in Yeast Genetics; a laboratory course manual: Cold Spring Harbor Laboratory, p.131-132(1990). S. cerevisiae S288C strain is inoculated in 10 ml of YPD media and cultured at 30°C overnight. The cultured cells are collected and suspended in 5 ml of sterilized water for washing. By discarding the supernatant, the yeast are collected again. A 0.2 ml of the mixture(2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl(pH 8), 1 mM EDTA), 0.2 ml of phenol/chloroform/isoamylalcohol (25/24/1 (v/v/v)) and 0.3 g of glass beads are added. After vortex mix for 3-4 minutes, two hundred μ l of TE buffer(10 mM Tris-HCl(pH 8), 1 mM EDTA) is added. Then the solution is centrifuged for 5 minutes, and the supernatant is transferred to another tube and 1 ml of ethanol is added. Then the solution is centrifuged again for 2 minutes. The resulted pellet is dissolved in 0.4 ml of TE buffer. Then, two μ l of RNase A(10 mg/ml) is added and the solution is stood for 5 minutes at 37°C. Then, ten μ l of 4 M ammonium acetate and 1 ml of ethanol are added. After mixing well, the solution is centrifuged for 2 minutes and the resulted pellet is recovered. After drying the pellet, it was dissolved with 50 μ l of TE buffer

to have total DNA of S. cerevisiae S288C strain. By this preparation procedure, 3.4 µg of total DNA was obtained.

(EXAMPLE 9) Isolation of the IPP isomerase gene of Saccharomyces cerevisiae by PCR method

Based on the nucleotide sequence of the IPP isomerase gene of S. cerevisiae reported in the aforementioned reference(Anderson, M. S., Muehlbacher, M., Street, I.P., Profitt, J., Poulter, C. D., "Isopentenyl diphosphate: dimethylallyl diphosphate isomerase - an improved purification of the enzyme and isolation of the gene from Saccharomyces cerevisiae", J. Biol. Chem., 264:19169-19175(1989)), the primers below were synthesized.

Primer No. 1 5'-TCGATGGGGGTTGCCTTTCTTTTTCGG-3'

Primer No. 2 5'-CGCGTTGTTATAGCATTCTATGAATTTGCC-3'

The procedure was designed to obtain PCR amplified IPP isomerase gene having TaqI sites at the upstream terminal and AccII region at the downstream terminal. Thirty cycles of PCR is performed with 200 ng of total DNA of S. cerevisiae and PfuDNA polymerase (STRATAGENE). To express the IPP isomerase gene obtained by PCR in E. coli, it is digested with both TaqI and AccII. Then, the gene was inserted into ClaI sites and SmaI sites of pBluescript KS+ vector. The resulted plasmid was named pSI1(Figure 8). This DNA derived from S. cerevisiae had a nucleotide sequence consisting of 1,058 bp (SEQUENCE ID No. 6), and contained a gene which encodes IPP isomerase consisting of 288 amino acids(corresponds from E to F in Figures 6).

(EXAMPLE 10) Increase of lycopene production amount by introducing the IPP isomerase gene

Into the lycopene-producing E. coli JM101 strain (abbreviated as L hereafter) which contains pACCRT-EIB(Figure 7), pSPORT1 vector, pRH1 plasmid containing the IPP isomerase gene of Phaffia rhodozyma, pHP11 plasmid containing the IPP isomerase gene of Haematococcus pluvialis or pSI1 plasmid containing the IPP isomerase gene of Saccharomyces cerevisiae(FIGURE 8) are introduced respectively. These E. coli transformants are then plated on the LB plate containing 150 µg/ml of ampicillin(Ap), 30 µg/ml of chloramphenicol(Cm) and 1 mM of IPTG, and cultured at 28°C overnight. The three strains, in which each IPP isomerase gene were introduced, showed deep reddish color due to lycopene production compared with the control (lycopene-producing E.coli) in which only vector is introduced. Furthermore, growth rate of the three strains on agar plates were faster than the control strains and they always showed larger colonies than those of the control during culture. It is considered that due to introduction and expression of the IPP isomerase gene, the upstream of the biosynthetic pathway up to FPP became more efficient(see FIGURE 1), and consequently, increase of FPP supply led to increase of lycopene. As for faster growth rate, it is also considered that due to increase of FPP, sufficient amount of the substrate can be supplied not only for lycopene production but also for the production of other membrane components derived from FPP, that is, FPP or GGPP binding protein, and these components are necessary for growth of E. coli.

Increase of lycopene production amount by E.coli carrying the IPP isomerase gene is also confirmed by liquid culture. After overnight shaking culture of the LB media(5 ml) containing both Ap and Cm at 28°C, 2 ml of the media is taken and transferred to

200 ml of 2YT culture media(1.6% bactotrypton, 1% yeast extract, 0.5% NaCl) containing Ap, Cp and 0.1 mM IPTG, and shaking culture is performed at 230 rpm, 28°C. Five ml each of the media is sampled several hours' intervals to determine growth rate and lycopene content. Growth rate is calculated from absorbance at 650 nm. Lycopene content is determined according to the following procedure. The cells collected by centrifugation, 2.5 ml of acetone is added to the cells and stand for 30 minutes. Vortex mix once in a while. After filtration, absorbance at 474 nm is measured to determine the lycopene content based on the absorbance 185.0 for 1 mM lycopene (light path: 1 cm). JASCO UVDEC-220B spectrophotometer is used. By using HPLC, it is confirmed that these strains actually produced lycopene and absorbance at 474 nm is attributable to lycopene. HPLC conditions are mentioned in EXAMPLE 11. The results are shown in Figure 9(growth curve) and Figure 10(lycopene production curve). As for the growth rate(Figure 9), there is no difference among any the strains including the control strains. This result is different from that obtained from culture plates. Probably, when the liquid culture is performed, even in the control strain which does not have exogenous IPP isomerase gene can grow rapidly, because the supply of the substrate for membrane components such as FPP and GGPP binding protein is enough compared to agar culture is done. In contrast, there is a big difference between the control strain having no exogenous IPP isomerase gene and the three exogenous IPP isomerase gene-carrying strains. During culture, the three strains always showed several times higher lycopene production amount compared with the control strain. Lycopene production amount per E. coli dry weight at 28 hr after

the start of the culture is shown in Figure 11. The three strains containing the IPP isomerase gene showed 3.6-4.5 times higher production than the control strain. Lycopene-producing E. coli containing pHP11 is able to produce 1.03 mg lycopene per 1g dry weight.

(EXAMPLE 11) Increase of β -carotene production amount by introducing the IPP isomerase gene

Into the β -carotene producing E. coli JM101 strain (abbreviated as β hereafter) which contains pACCAR16 Δ crtX (FIGURE 7), either pSPORT1 vector or pRH1 plasmid containing the IPP isomerase gene of Phaffia rhodozyma is introduced separately. After overnight shaking culture of the LB media (5 ml) containing both Ap and Cm at 28°C, 1 ml of the media is taken and transferred to 100 ml of 2YT media containing Ap, Cm and 0.1 mM IPTG, and shaking culture is performed at 230 rpm at 28°C for 28 hr. The bacteria are collected by centrifugation and washed with 0.85% NaCl. After washing, the bacteria are suspended in 40 ml of acetone and allowed to stand for 30 minutes. Vortex mix once in a while. After filtration, absorbance at 454 nm is measured to determine β -carotene content based on the absorbance 134.4 for 1 mM β -carotene (light path: 1 cm). The result is shown in FIGURE 11. β -Carotene producing E. coli containing pRH1 produced 709 μ g of β -carotene per 1g dry weight. This amount is 1.5 times higher than the control.

By using HPLC on the above acetone extract, it is confirmed that these strains actually produced β -carotene and absorbance at 454 nm is attributable to β -carotene. Novapack HR 6 μ C18 (3.9 x 300 mm, Waters) is used as a column. Acetonitrile/methanol/2-

propanol(90/6/4(v/v/v)) is used as an elution solvent. A photodiode array detector 996(Waters) is used to monitor an elution profile. The results showed that almost 100% of a peak appeared in a visible spectrum is β -carotene. As the β -carotene standard preparation, chemically synthesized β -carotene (Sigma) is used.

(EXAMPLE 12) Increase of phytoene production amount by introducing the IPP isomerase gene

Into the phytoene producing E. coli JM101 strain (abbreviated as P hereafter) which contains pACCRT-EB(FIGURE 7), any of pSPORT1 vector , pRH1 plasmid containing the IPP isomerase gene of Phaffia rhodozyma or pHP11 plasmid containing the IPP isomerase gene of Haematococcus pluvialis is introduced separately. After overnight shaking culture of the LB media(5 ml) containing both Ap and Cm at 28°C, 1 ml of the media is taken and transferred to 100 ml of 2YT media containing Ap, Cm and 0.1 mM IPTG, and shaking culture is performed at 230 rpm at 28°C for 28 hr. The bacteria are collected by centrifugation and washed with 0.85% NaCl. After washing, the bacteria are suspended in 40 ml of acetone and allowed to stand for 30 minutes. Vortex mix once in a while. After filtration and drying by rotary evaporator, partition is performed with 40 ml of petroleum ether and water. Absorbance of the ether layer at 286 nm is measured to determine phytoene content based on the absorbance 41.2 for 1 mM phytoene (light path: 1 cm). As HPLC analysis described in EXAMPLE 11 showed that 70% of the absorbance at 286 nm is attributable to phytoene, an and also actual phytoene content is adjusted to 70% of the above value. The result is shown in

Leu	Leu	Leu	Gln	Arg	Arg	Ala	Asp	Glu	Lys	Ile	Thr	Phe	Pro	Gly	Met
				85				90					95		
Trp	Thr	Asn	Thr	Cys	Cys	Ser	His	Pro	Leu	Ser	Ile	Lys	Gly	Glu	Val
		100					105				110				
Glu	Glu	Glu	Asn	Gln	Ile	Gly	Val	Arg	Arg	Ala	Ala	Ser	Arg	Lys	Leu
		115				120				125					
Glu	His	Glu	Leu	Gly	Val	Pro	Thr	Ser	Ser	Thr	Pro	Pro	Asp	Ser	Phe
	130				135					140					
Thr	Tyr	Leu	Thr	Arg	Ile	His	Tyr	Leu	Ala	Pro	Ser	Asp	Gly	Leu	Trp
145				150				155				160			
Gly	Glu	His	Glu	Ile	Asp	Tyr	Ile	Leu	Phe	Ser	Thr	Thr	Pro	Thr	Glu
			165				170					175			
His	Thr	Gly	Asn	Pro	Asn	Glu	Val	Ser	Asp	Thr	Arg	Tyr	Val	Thr	Lys
		180					185				190				
Pro	Glu	Leu	Gln	Ala	Met	Phe	Glu	Asp	Glu	Ser	Asn	Ser	Phe	Thr	Pro
	195				200					205					
Trp	Phe	Lys	Leu	Ile	Ala	Arg	Asp	Phe	Leu	Phe	Gly	Trp	Trp	Asp	Gln
	210				215				220						
Leu	Leu	Ala	Arg	Arg	Asn	Glu	Lys	Gly	Glu	Val	Asp	Ala	Lys	Ser	Leu
225				230				235				240			
Glu	Asp	Leu	Ser	Asp	Asn	Lys	Val	Trp	Lys	Met	***				
			245					250							

SEQUENCE ID No.: 2

LENGTH: 259

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULAR TYPE: peptide

SEQUENCE:

met	Gln	Leu	Leu	Ala	Glu	Asp	Arg	Thr	Asp	His	Met	Arg	Gly	Ala	Ser
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Thr	Trp	Ala	Gly	Gly	Gln	Ser	Gln	Asp	Glu	Leu	Met	Leu	Lys	Asp	Glu
		20				25				30					
Cys	Ile	Leu	Val	Asp	Ala	Asp	Asp	Asn	Ile	Thr	Gly	His	Val	Ser	Lys
	35					40				45					

Leu	Glu	Cys	His	Lys	Phe	Leu	Pro	His	Gln	Pro	Ala	Gly	Leu	Leu	His
50						55				60					
Arg	Ala	Phe	Ser	Val	Phe	Leu	Phe	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Leu
65					70				75				80		
Gln	Gln	Arg	Ala	Arg	Ser	Lys	Ile	Thr	Phe	Pro	Ser	Val	Trp	Thr	Asn
			85					90				95			
Thr	Cys	Cys	Ser	His	Pro	Leu	His	Gly	Gln	Thr	Pro	Asp	Glu	Val	Asp
			100				105					110			
Gln	Leu	Ser	Gln	Val	Ala	Asp	Gly	Thr	Val	Pro	Gly	Ala	Lys	Ala	Ala
		115					120					125			
Ala	Ile	Arg	Lys	Leu	Glu	His	Glu	Leu	Gly	Ile	Pro	Ala	His	Gln	Leu
		130					135				140				
Pro	Ala	Ser	Ala	Phe	Arg	Phe	Leu	Thr	Arg	Leu	His	Tyr	Cys	Ala	Ala
145				150					155				160		
Asp	Val	Gln	Pro	Ala	Ala	Thr	Gln	Ser	Ala	Leu	Trp	Gly	Glu	His	Glu
				165				170				175			
Met	Asp	Tyr	Ile	Leu	Phe	Ile	Arg	Ala	Asn	Val	Thr	Leu	Ala	Pro	Asn
		180					185				190				
Pro	Asp	Glu	Val	Asp	Glu	Val	Arg	Tyr	Val	Thr	Gln	Glu	Glu	Leu	Arg
		195					200				205				
Gln	Met	Met	Gln	Pro	Asp	Asn	Gly	Leu	Gln	Trp	Ser	Pro	Trp	Phe	Arg
		210					215				220				
Ile	Ile	Ala	Ala	Arg	Phe	Leu	Glu	Arg	Trp	Trp	Ala	Asp	Leu	Asp	Ala
225				230					235				240		
Ala	Leu	Asn	Thr	Asp	Lys	His	Glu	Asp	Trp	Gly	Thr	Val	His	His	Ile
				245				250				255			
Asn	Glu	Ala	***												

SEQUENCE ID No.: 3

LENGTH: 288

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULAR TYPE: peptide

SEQUENCE:

Met Thr Ala Asp Asn Asn Ser Met Pro His Gly Ala Val Ser Ser Tyr

SEQUENCE ID No.: 4

LENGTH: 1099

SEQUENCE TYPE: nucleic acid

STRANDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: cDNA

ORIGIN

ORGANISM: *Phaffia rhodozyma*

STRAIN: ATCC 24230

SEQUENCE CHARACTERISTIC

CHARACTERISTIC CODE: CDS

LOCATION: 99..851

DETERMINATION METHOD: E

SEQUENCE:

CCCACGCGTC CGCACATCTC GCATATATCA CTTTCCTCCT TCCAGAACAA GTTCTGAGTC 60

AACCGAAAAG AAAGAAGGCA GAGGAAAATA TATTCTAG ATG TCC ATG CCC AAC ATT 1:

Met Ser Met Pro Asn Ile

5

GTT CCC CCC GCC GAG GTC CGA ACC GAA GGA CTC AGT TTA GAA GAG TAC 164

Val Pro Pro Ala Glu Val Arg Thr Glu Gly Leu Ser Leu Glu Glu Tyr

10

15

20

GAT GAG GAG CAG GTC AGG CTG ATG GAG GAG CGA TGT ATT CTT GTT AAC 212

Asp Glu Glu Gln Val Arg Leu Met Glu Glu Arg Cys Ile Leu Val Asn

25

30

35

CCG GAC GAT GTG GCC TAT GGA GAG GCT TCG AAA AAG ACC TGC CAC TTG 260

Pro Asp Asp Val Ala Tyr Gly Glu Ala Ser Lys Lys Thr Cys His Leu

40

45

50

ATG TCC AAC ATC AAC GCG CCC AAG GAC CTC CTC CAC CGA GCA TTC TCC 308

Met Ser Asn Ile Asn Ala Pro Lys Asp Leu Leu His Arg Ala Phe Ser

55

60

65

70

GTG TTT CTC TTC CGC CCA TCG GAC GGA GCA CTC CTG CTT CAG CGA AGA 356

Val Phe Leu Phe Arg Pro Ser Asp Gly Ala Leu Leu Leu Gln Arg Arg

75

80

85

GCG GAC GAG AAG ATT ACG TTC CCT GGA ATG TGG ACC AAC ACG TGT TGC 404
 Ala Asp Glu Lys Ile Thr Phe Pro Gly Met Trp Thr Asn Thr Cys Cys
 90 95 100
 AGT CAT CCT TTG AGC ATC AAG GGC GAG GTT GAA GAG GAG AAC CAG ATC 452
 Ser His Pro Leu Ser Ile Lys Gly Glu Val Glu Glu Glu Asn Gln Ile
 105 110 115
 GGT GTT CGA CGA GCT GCG TCC CGA AAG TTG GAG CAC GAG CTT GGC GTG 500
 Gly Val Arg Arg Ala Ala Ser Arg Lys Leu Glu His Glu Leu Gly Val
 120 125 130
 CCT ACA TCG TCG ACT CCG CCC GAC TCG TTC ACC TAC CTC ACT AGG ATA 548
 Pro Thr Ser Ser Thr Pro Pro Asp Ser Phe Thr Tyr Leu Thr Arg Ile
 135 140 145 150
 CAT TAC CTC GCT CCG AGT GAC GGA CTC TGG GGA GAA CAC GAG ATC GAC 596
 His Tyr Leu Ala Pro Ser Asp Gly Leu Trp Gly Glu His Glu Ile Asp
 155 160 165
 TAC ATT CTC TTC TCA ACC ACA CCT ACA GAA CAC ACT GGA AAC CCT AAC 644
 Tyr Ile Leu Phe Ser Thr Thr Pro Thr Glu His Thr Gly Asn Pro Asn
 170 175 180
 GAA GTC TCT GAC ACT CGA TAT GTC ACC AAG CCC GAG CTC CAG GCG ATG 692
 Glu Val Ser Asp Thr Arg Tyr Val Thr Lys Pro Glu Leu Gln Ala Met
 185 190 195
 TTT GAG GAC GAG TCT AAC TCA TTT ACC CCT TGG TTC AAG TTG ATT GCC 740
 Phe Glu Asp Glu Ser Asn Ser Phe Thr Pro Trp Phe Lys Leu Ile Ala
 200 205 210
 CGA GAC TTC CTG TTT GGC TGG TGG GAT CAA CTT CTC GCC AGA CGA AAT 788
 Arg Asp Phe Leu Phe Gly Trp Trp Asp Gln Leu Leu Ala Arg Arg Asn
 215 220 225 230
 GAA AAG GGT GAG GTC GAT GCC AAA TCG TTG GAG GAT CTC TCG GAC AAC 836
 Glu Lys Gly Glu Val Asp Ala Lys Ser Leu Glu Asp Leu Ser Asp Asn
 235 240 245
 AAA GTC TGG AAG ATG TAGTCGACC CTTCTTTCTG TACAGTCATC TCAGTTCGCC 890
 Lys Val Trp Lys Met ***
 250
 TGTGTTGTTGC TTGCTTCTTG CTCTTCTTTC TATATATCTT TTTTCTTGCC TGGGTAGACT 95
 TGATCTTTCT ACATAGCATA CGCATAACATA CATAAACTCT ATTTCTTGTT CTTTATCTCT 10
 CTTCTAAGGG AATCTTCAAG ATCAATTTCT TTTTGGGCTA CAACATTTCA GATCAATGTT 10

GCTTTTCAGA CTACAAAAA AAAAAAAAAA 1099

SEQUENCE ID No.: 5

LENGTH: 1074

SEQUENCE TYPE: nucleic acid

STRANDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: cDNA

ORIGIN

ORGANISM: Haematococcus pluvialis

STRAIN: NIES-144

SEQUENCE CHARACTERISTIC

CHARACTERISTIC CODE: CDS

LOCATIION: 145..921

DETERMINATION METHOD: E

SEQUENCE:

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AGAGGCCTCA CGCATTTCCC CCGCGTGAAC TCCGCGCAGC AGCCCAGCTG TGCACACGCG 120
CGACTCCAGT TTAGGCCCCAG AAGC ATG CAG CTG CTT GCC GAG GAC CGC ACA GAC 1
Met Gln Leu Leu Ala Glu Asp Arg Thr Asp
5 10
CAT ATG AGG GGT GCA AGT ACC TGG GCA GGC GGG CAG TCG CAG GAT GAG 222
His Met Arg Gly Ala Ser Thr Trp Ala Gly Gly Gln Ser Gln Asp Glu
15 20 25
CTG ATG CTG AAG GAC GAG TGC ATC TTG GTG GAT GCT GAC GAC AAC ATT 270
Leu Met Leu Lys Asp Glu Cys Ile Leu Val Asp Ala Asp Asp Asn Ile
30 35 40
ACA GGC CAT GTC AGC AAG CTG GAG TGC CAC AAG TTC CTA CCA CAT CAG 318
Thr Gly His Val Ser Lys Leu Glu Cys His Lys Phe Leu Pro His Gln
45 50 55
CCT GCA GGC CTG CTG CAC CGG GCC TTC TCT GTA TTC CTG TTT GAC GAC 366
```

Pro	Ala	Gly	Leu	Leu	His	Arg	Ala	Phe	Ser	Val	Phe	Leu	Phe	Asp	Asp	
60					65				70							
CAG	GGG	CGA	CTG	CTG	CTG	CAA	CAG	CGT	GCA	CGA	TCA	AAA	ATC	ACA	TTC	414
Gln	Gly	Arg	Leu	Leu	Leu	Gln	Gln	Arg	Ala	Arg	Ser	Lys	Ile	Thr	Phe	
75				80				85				90				
CCC	AGT	GTG	TGG	ACC	AAC	ACC	TGC	TGC	AGC	CAC	CCT	CTA	CAT	GGG	CAG	462
Pro	Ser	Val	Trp	Thr	Asn	Thr	Cys	Cys	Ser	His	Pro	Leu	His	Gly	Gln	
			95				100				105					
ACC	CCA	GAT	GAG	GTG	GAC	CAA	CTA	AGC	CAG	GTG	GCC	GAC	GGC	ACA	GTA	510
Thr	Pro	Asp	Glu	Val	Asp	Gln	Leu	Ser	Gln	Val	Ala	Asp	Gly	Thr	Val	
		110				115				120						
CCT	GGC	GCA	AAG	GCT	GCT	GCC	ATC	CGC	AAG	TTG	GAG	CAC	GAG	CTG	GGG	558
Pro	Gly	Ala	Lys	Ala	Ala	Ala	Ile	Arg	Lys	Leu	Glu	His	Glu	Leu	Gly	
	125				130			135								
ATA	CCA	GCG	CAC	CAG	CTG	CCG	GCC	AGC	GCG	TTT	CGC	TTC	CTC	ACG	CGT	606
Ile	Pro	Ala	His	Gln	Leu	Pro	Ala	Ser	Ala	Phe	Arg	Phe	Leu	Thr	Arg	
	140			145			150									
TTG	CAC	TAC	TGC	GCC	GCG	GAC	GTG	CAG	CCG	GCT	GCG	ACA	CAA	TCA	GCA	654
Leu	His	Tyr	Cys	Ala	Ala	Asp	Val	Gln	Pro	Ala	Ala	Thr	Gln	Ser	Ala	
155			160			165		170								
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Leu	Trp	Gly	Glu	His	Glu	Met	Asp	Tyr	Ile	Leu	Phe	Ile	Arg	Ala	Asn	
		175				180		185								
GTC	ACC	CTT	GCG	CCC	AAC	CCT	GAC	GAG	GTG	GAC	GAA	GTC	AGG	TAC	GTG	750
Val	Thr	Leu	Ala	Pro	Asn	Pro	Asp	Glu	Val	Asp	Glu	Val	Arg	Tyr	Val	
	190					195		200								
ACG	CAG	GAG	GAG	CTG	CGG	CAG	ATG	ATG	CAG	CCG	GAC	AAT	GGG	TTG	CAA	798
Thr	Gln	Glu	Glu	Leu	Arg	Gln	Met	Met	Gln	Pro	Asp	Asn	Gly	Leu	Gln	
	205			210			215									
TGG	TCG	CCG	TGG	TTT	CGC	ATC	ATC	GCC	GCG	CGC	TTC	CTT	GAG	CGC	TGG	846
Trp	Ser	Pro	Trp	Phe	Arg	Ile	Ile	Ala	Ala	Arg	Phe	Leu	Glu	Arg	Trp	
	220			225			230									
TGG	GCT	GAC	CTA	GAC	GCG	GCC	CTG	AAC	ACT	GAC	AAA	CAC	GAG	GAT	TGG	894
Trp	Ala	Asp	Leu	Asp	Ala	Ala	Leu	Asn	Thr	Asp	Lys	His	Glu	Asp	Trp	
235			240			245		250								
GGA	ACG	GTG	CAT	CAC	ATC	AAC	GAA	GCG	TGA	AAACAG	AAGCTGTAGG					940
Gly	Thr	Val	His	His	Ile	Asn	Glu	Ala	***							
			255													
ATGTCAAGAC	ACGTCATGAG	GGGGCTTGGC	ATCTTGCGCG	CTTCGTATCT	CTTTTTACTG											10

AGACTGAACC TGCAGCTGGA GACAATGGTG AGCCCAATTC AACTTTCCGC TGC ACTGGAA 10

AAAAAAAAAA AAAA 1074

SEQUENCE ID No.: 6

LENGTH: 1058

SEQUENCE TYPE: nucleic acid

STRANDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: genomic DNA

ORIGIN

ORGANISM: *Saccharomyces cerevisiae*

STRAIN: S288C

SEQUENCE CHARACTERISTIC

CHARACTERISTIC CODE: CDS

LOCATIION: 187..1050

DETERMINATION METHOD: S

SEQUENCE:

TCGATGGGGG TTGCCTTTCT TTTTCGGTCT TAACTCCATT TATATTTATT TATTCATTTT 60

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ATATTAGAGT ATTCGTATTT GGAATACAGG AAGAGTAAAA ATAAGCCAAA AATTCATTAC 18

ACCTCA ATG ACT GCC GAC AAC AAT AGT ATG CCC CAT GGT GCA GTA TCT AGT 2

Met Thr Ala Asp Asn Asn Ser Met Pro His Gly Ala Val Ser Ser

5

10

15

TAC GCC AAA TTA GTG CAA AAC CAA ACA CCT GAA GAC ATT TTG GAA GAG 279

Tyr Ala Lys Leu Val Gln Asn Gln Thr Pro Glu Asp Ile Leu Glu Glu

20

25

30

TTT CCT GAA ATT ATT CCA TTA CAA CAA AGA CCT AAT ACC CGA TCT AGT 327

Phe Pro Glu Ile Ile Pro Leu Gln Gln Arg Pro Asn Thr Arg Ser Ser

															35																40																45																															
GAG	ACG	TCA	AAT	GAC	GAA	AGC	GGA	GAA	ACA	TGT	TTT	TCT	GGT	CAT	GAT	375																																																														
Glu	Thr	Ser	Asn	Asp	Glu	Ser	Gly	Glu	Thr	Cys	Phe	Ser	Gly	His	Asp																																																															
															50																55																60																															
GAG	GAG	CAA	ATT	AAG	TTA	ATG	AAT	GAA	AAT	TGT	ATT	GTT	TTG	GAT	TGG	423																																																														
Glu	Glu	Gln	Ile	Lys	Leu	Met	Asn	Glu	Asn	Cys	Ile	Val	Leu	Asp	Trp																																																															
															65																70																75																															
GAC	GAT	AAT	GCT	ATT	GGT	GCC	GGT	ACC	AAG	AAA	GTT	TGT	CAT	TTA	ATG	471																																																														
Asp	Asp	Asn	Ala	Ile	Gly	Ala	Gly	Thr	Lys	Lys	Val	Cys	His	Leu	Met																																																															
															80																85																90																95															
GAA	AAT	ATT	GAA	AAG	GGT	TTA	CTA	CAT	CGT	GCA	TTC	TCC	GTC	TTT	ATT	519																																																														
Glu	Asn	Ile	Glu	Lys	Gly	Leu	Leu	His	Arg	Ala	Phe	Ser	Val	Phe	Ile																																																															
															100																105																110																															
TTC	AAT	GAA	CAA	GGT	GAA	TTA	CTT	TTA	CAA	CAA	AGA	GCC	ACT	GAA	AAA	567																																																														
Phe	Asn	Glu	Gln	Gly	Glu	Leu	Leu	Leu	Gln	Gln	Arg	Ala	Thr	Glu	Lys																																																															
															115																120																125																															
ATA	ACT	TTC	CCT	GAT	CTT	TGG	ACT	AAC	ACA	TGC	TGC	TCT	CAT	CCA	CTA	615																																																														
Ile	Thr	Phe	Pro	Asp	Leu	Trp	Thr	Asn	Thr	Cys	Cys	Ser	His	Pro	Leu																																																															
															130																135																140																															
TGT	ATT	GAT	GAC	GAA	TTA	GGT	TTG	AAG	GGT	AAG	CTA	GAC	GAT	AAG	ATT	663																																																														
Cys	Ile	Asp	Asp	Glu	Leu	Gly	Leu	Lys	Gly	Lys	Leu	Asp	Asp	Lys	Ile																																																															
															145																150																155																															
AAG	GGC	GCT	ATT	ACT	GCG	GCG	GTG	AGA	AAA	CTA	GAT	CAT	GAA	TTA	GGT	711																																																														
Lys	Gly	Ala	Ile	Thr	Ala	Ala	Val	Arg	Lys	Leu	Asp	His	Glu	Leu	Gly																																																															
															160																165																170																175															
ATT	CCA	GAA	GAT	GAA	ACT	AAG	ACA	AGG	GGT	AAG	TTT	CAC	TTT	TTA	AAC	759																																																														
Ile	Pro	Glu	Asp	Glu	Thr	Lys	Thr	Arg	Gly	Lys	Phe	His	Phe	Leu	Asn																																																															
															180																185																190																															
AGA	ATC	CAT	TAC	ATG	GCA	CCA	AGC	AAT	GAA	CCA	TGG	GGT	GAA	CAT	GAA	807																																																														
Arg	Ile	His	Tyr	Met	Ala	Pro	Ser	Asn	Glu	Pro	Trp	Gly	Glu	His	Glu																																																															
															195																200																205																															
ATT	GAT	TAC	ATC	CTA	TTT	TAT	AAG	ATC	AAC	GCT	AAA	GAA	AAC	TTG	ACT	855																																																														
Ile	Asp	Tyr	Ile	Leu	Phe	Tyr	Lys	Ile	Asn	Ala	Lys	Glu	Asn	Leu	Thr																																																															
															210																215																220																															
GTC	AAC	CCA	AAC	GTC	AAT	GAA	GTT	AGA	GAC	TTC	AAA	TGG	GTT	TCA	CCA	903																																																														
Val	Asn	Pro	Asn	Val	Asn	Glu	Val	Arg	Asp	Phe	Lys	Trp	Val	Ser	Pro																																																															
															225																230																235																															
AAT	GAT	TTG	AAA	ACT	ATG	TTT	GCT	GAC	CCA	AGT	TAC	AAG	TTT	ACG	CCT	951																																																														
Asn	Asp	Leu	Lys	Thr	Met	Phe	Ala	Asp	Pro	Ser	Tyr	Lys	Phe	Thr	Pro																																																															

240	245	250	255	
TGG TTT AAG ATT ATT TGC GAG AAT TAC TTA TTC AAC TGG TGG GAG CAA	999			
Trp Phe Lys Ile Ile Cys Glu Asn Tyr Leu Phe Asn Trp Trp Glu Gln				
260	265	270		
TA GAT GAC CTT TCT GAA GTG GAA AAT GAC AGG CAA ATT CAT AGA ATG	1047			
Leu Asp Asp Leu Ser Glu Val Glu Asn Asp Arg Gln Ile His Arg Met				
275	280	285		
CTA TAA CAACG	1058			
Leu ***				

[Effect of the Invention]

The present invention provides a DNA chain which can significantly increase carotenoid production in biosynthesis of carotenoid by microorganisms and a method to obtain several times higher carotenoid production amount by introducing and expressing said DNA chain into carotenoid-producing microorganisms. It is expected that said DNA chain can be applicable to increase production in microorganisms not only for carotenoids but also for terpenoids and so forth which require same substrate(FPP) as carotenoids.

[Brief Description of the Drawings]

FIGURE 1 shows the isoprene common biosynthetic pathway from HMG-CoA to FPP.

FIGURE 2 shows the carotenoid biosynthetic pathway, and the functions of the carotenoid synthesis genes of Erwinia uredovora, the non-photosynthetic bacterium.

FIGURE 3 shows the carotenoid biosynthetic pathway, and the functions of the carotenoid synthesis genes of Agrobacterium aurantiacum, the marine bacterium. The solid line shows major biosynthetic pathway and the dotted line shows minor one.

FIGURE 4 shows the nucleotide sequence of the IPP isomerase gene and the amino acid sequence of the polypeptide encoded by

said gene of Phaffia rhodozyma, the astaxanthin-producing yeast. In the Figure, the sequence from mark A to B shows the open reading frame encoding the polypeptide consisting of 251 amino acids.

FIGURE 5 shows the nucleotide sequence of the IPP isomerase gene and the amino acid sequence of the polypeptide encoded by said gene of Haematococcus pluvialis, the astaxanthin-producing green alga. In the Figure, the sequence from mark C to D shows the open reading frame encoding the polypeptide consisting of 259 amino acids.

FIGURE 6 shows the nucleotide sequence of the IPP isomerase gene and the amino acid sequence of the polypeptide encoded by said gene of Saccharomyces cerevisiae, the yeast for laboratory use. In the Figure, the sequence from mark E to F shows the open reading frame encoding the polypeptide consisting of 288 amino acids.

FIGURE 7 shows the plasmids containing the carotenoid biosynthesis genes of Erwinia uredovora, the non-photosynthetic bacterium.

FIGURE 8 shows the plasmids containing the IPP isomerase gene of Phaffia rhodozyma, Haematococcus pluvialis, or Saccharomyces cerevisiae.

FIGURE 9 shows the growth curve in the culture broth of the lycopene producing E. coli strains(L:). In the Figure, "control" means the E. coli strain having no exogenous IPP isomerase gene.

FIGURE 10 shows the lycopene production curve in the culture broth of the lycopene producing E. coli strains(L:). In the Figure, "control" means the E. coli strain having no exogenous IPP isomerase gene.

FIGURE 11 shows production of lycopene(L:), β -carotene(β :) and phytoene(P:) in the cultured cells of the E. coli strains. In the Figure, "control" means the E. coli strain having no exogenous IPP isomerase gene.

[Name of Document] Abstract

[Abstract]

[Constitution]

[Effect]

[Selected Figure] None

A DNA chain having characteristic of increasing carotenoid production, and containing the nucleotide sequence which encodes the polypeptide having the substantially same amino acid sequence described in SEQUENCE ID No. 1 or 2, or a DNA chain which hybridizes with said DNA chain, and a method for production for carotenoid characterized by introducing said DNA chain into the carotenoid-producing microorganisms, culturing said transformed microorganism and increasing carotenoid content in the culture broth and cells .